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Caterpillar- and Salivary-Specific Modification of Plant Proteins

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Though there is overlap, plant responses to caterpillar herbivory show distinct variations from mechanical wounding. In particular, effectors in caterpillar oral secretions modify wound-associated plant responses. Previous studies have focused on transcriptional and protein abundance differences in response to caterpillar herbivory. This study investigated *Spodoptera exigua* caterpillar-specific post-translational modification of *Arabidopsis thaliana* soluble leaf proteins by liquid chromatography/electrospray ionization/mass spectroscopy/mass spectroscopy (LC/ESI/MS/MS). Given that caterpillar labial saliva contains oxidoreductases, such as glucose oxidase, particular attention was paid to redox-associated modifications, such as the oxidation of protein cysteine residues. Caterpillar- and saliva-specific protein modifications were observed. Differential phosphorylation of the jasmonic acid biosynthetic enzyme, lipoxygenase 2, and a chaperonin protein is seen in plants fed upon by caterpillars with intact salivary secretions compared to herbivory by larvae with impaired labial salivary secretions. Often a systemic suppression of photosynthesis is associated with caterpillar herbivory. Of the five proteins modified in a caterpillar-specific manner (a transcription repressor, a DNA-repair enzyme, PS I P700, Rubisco and Rubisco activase), three are associated with photosynthesis. Oxidative modifications are observed, such as caterpillar-specific denitrosylation of Rubisco activase and chaperonin, cysteine oxidation of Rubisco, DNA-repair enzyme, and chaperonin and caterpillar-specific 4-oxo-2-nonenal modification of the DNA-repair enzyme.

Keywords: caterpillar • covalent modification • cysteine oxidation • liquid chromatography/electrospray ionization/mass spectroscopy/mass spectroscopy • nitrosylation • phosphorylation • proteomics

Introduction

Even though chewing herbivores wound the plant during feeding, temporal, quantitative and qualitative differences in plant responses are often observed in response to caterpillar herbivory compared to mechanical damage.[1−3] Caterpillar-specific plant responses begin immediately; even before the first bite is taken, the plant may detect caterpillar footsteps along the leaf surface and begin to mount a response.[4] Effectors in caterpillar oral secretions also modify the plants' induced responses. Regurgitant-associated fatty acid-amino acid conjugates amplify jasmonic acid (JA)-dependent induced resistance.[5,6] In comparison, labial salivary effectors have been implicated in a caterpillar design to circumvent the induction of plant defenses.[7−10] Caterpillar salivary glucose oxidase (GOX) prevents the induction of defensive nicotine production in tobacco plants;[7,8] the specific mechanism is unknown but is believed to involve hydrogen peroxide-associated changes in cellular redox potential.[5,9]

Both wounding and Noctuid caterpillar-associated labial salivary enzymes change the cellular redox potential by affecting levels of reactive oxygen species, particularly hydrogen peroxide.[5,8,11−13] In numerous plant species, wound-induced signal transduction pathways activate membrane-bound NADPH oxidases that lead to localized, apoplastic hydrogen peroxide accumulation.[11,12] However, caterpillar-specific effects on hydrogen peroxide accumulation are also noted. In lima bean (*Phaseolus lunatus* L.), higher hydrogen peroxide levels accumulate approximately 500 μm from the site of damage by herbivory with *Spodoptera littoralis* Boisduval caterpillars compared to mechanical wounding.[11] This caterpillar-associated hydrogen peroxide reflects the presence of salivary oxidoreductases secreted onto the plant tissues during feeding.[14−17] Though some salivary-associated enzymes, such as ascorbate peroxidase, may utilize hydrogen peroxide their activity appears to be minor compared with other labial salivary oxidoreductases, such as glucose oxidase, that leads to the production of hydrogen peroxide.[5,14,15] In addition, other salivary enzymes may impact cellular redox potential indirectly: the Noctuid-

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associated enzyme lysozyme cleaves bacterial cell wall peptidoglycans generating fragments that can be recognized by plants and lead to elevated salicylic acid and hydrogen peroxide levels.[18,19]

In biological systems, hydrogen peroxide plays a dual role. At excessive concentrations, this reactive molecule leads to cell damage by reacting with DNA, protein, and lipids leading to mutations and impairing function.[20,21] However, at lower, controlled concentrations, hydrogen peroxide is an important signaling molecule implicated in plant development, physiology, and stress responses.[22,23] One way hydrogen peroxide exerts its subtle effects is through protein modification. Post-translational covalent modifications of proteins is an often reversible change resulting in a change of protein activity, regulation, localization, protein−protein interactions, or stability.[24,25] As the cellular oxidation level increases, protein cysteine modification by glutathionylation, nitrosylation, hydrogen peroxide-mediated oxidation, and lipid peroxidation increases.[26,27] As cellular hydrogen peroxide levels increase, oxidation of the cysteine thiol (Cys-SH) groups generates the sulphenic acid form (Cys-SOH). The susceptibility and, therefore, specificity of a particular cysteine thiolate (Cys-S^-) to oxidation not only reflects its low pK_a but also its neighboring amino acids and cellular environment.[21,26,28] The cysteine sulphenic acid can further react with a cysteine thiol to generate a disulfide bridge (Cys-S-S-Cys).[29] However, in addition to the hydrogen peroxide-mediated oxidation of the cysteine thiolate (Cys-S^-) to the sulphenic acid (Cys-SOH), under increasing oxidative conditions, there may be further oxidation of the oxidation of the sulphenic acid (Cys-SOH) to the sulphinic acid (Cys-SO_2H) or even the sulfonic acid (Cys-SO_3H). The sulphinic and sulfonic acid forms of cysteine are more stable oxidative forms. Cysteine sulfinic acid can be reduced to the sulphenic acid form by enzymes such as sulphiredoxin.[30,31] The sulphenic acid can also be further reduced by thioredoxin and glutaredoxin.[32] On the other hand, the sulfonic acid form of cysteine is considered to be irreversible.[33] Therefore, in a biological context, cysteine oxidation allows key proteins to be in tune with the changing cellular conditions which reflect development or environmental cues. For example, 2-Cys peroxiredoxin is a key player in prokaryotic and eukaryotic redox signaling pathways;[34] the active form of this enzyme is dimeric stabilized by a disulfide bridge. In response to stress and increasing cellular hydrogen peroxide, the Cys_{61} thiolate (Cys-S^-) in *Escherichia coli* 2-Cys peroxiredoxin is oxidized to the sulfenic acid (Cys-SOH) which forms a disulfide bridge with the Cys_{95} thiol (Cys-SH) on a neighboring 2-Cys peroxiredoxin protein forming a stable active dimer.[35] However, under high oxidative stress, the increased hydrogen peroxide results in the "over oxidation" of the cysteine to the sulfinic acid (Cys-SO_2H) form resulting in an inactive enzyme that is unable to form a dimer.[34]

Elevated hydrogen peroxide levels also perturb the ascorbate/glutathione cycle which acts as a major cellular redox buffer.[22,36] The resultant increase in oxidized glutathione (GSSG) can react with the cysteine sulfenic acid resulting in glutathionylation (Cys-SSG).[20,21] Reactive nitrogen species, such as nitric oxide (NO), can react directly or indirectly via GSH and NO-generated nitrosoglutathione (GSNO) with cysteine thiols (Cys-SH) resulting in protein nitrosylation (Cys-SNO).[26,27] Another indicator of oxidative stress is protein modification by lipid peroxidation.[37,38] Either ROS-activated lipoxygenases or hydrogen peroxide directly can lead to the oxidation of membrane-bound lipids generating highly reactive peroxyl radicals that form Michael-type adducts with cysteine sulfhydryl groups (Cys-SH). Through hydrogen peroxide-mediated changes in cellular redox potential or through its direct action on cysteine modification, redox-sensitive kinases or phosphatase may also be activated leading to phosphorylation-related covalent modification of downstream target proteins.[39−41]

Caterpillar labial saliva contains numerous oxidoreductases and as-of-yet uncharacterized effectors that are secreted onto plant tissues during feeding and believed to be responsible for undermining plant induced defenses.[7,10,15] Since the product of one of the major Noctuid salivary protein, glucose oxidase, is hydrogen peroxide that can lead to activation of signaling pathways through cysteine oxidation, this study investigates caterpillar-specific protein modifications. Previous studies have investigated the transcriptional and proteomic responses of plants to caterpillar herbivory;[1,9,42,43] however, few studies have focused on the identification of caterpillar-specific modified proteins. *Arabidopsis thaliana* (L.) Heynh. plants were subjected to herbivory by fourth instar *Spodoptera exigua* Hübner caterpillars with intact or impaired labial salivary secretions. Soluble proteins were extracted and analyzed by liquid chromatography/electrospray ionization/mass spectroscopy/mass spectroscopy (LC/ESI/MS/MS) and identified against the *Arabidopsis* database using conservative criteria. Seven proteins were identified as being covalently modified in a caterpillar- or salivary-dependent manner. These modifications include phosphorylation, nitrosylation, lipid peroxidation, and cysteine oxidation.

Experimental Section

Plant Material and Growth Conditions. After cold stratification for 3 days, seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia-0) were sown in Promix BX (Premier Horticulture Inc.) and grown in a phytorium growth cabinet under defined conditions (day/night temperature: 22 °C/18 °C; photoperiod 16 h light/8 h dark; irradiance set at 150 μmol m^{-2} s^{-1} with fluorescent and incandescent lights). Plants were bottom-watered as needed in weak fertilizer (one-sixth dilution of 20−20−20 NPK). At approximately 4 weeks, seedlings were transplanted individually to 16 cm pots. Vegetative six week old seedlings grown to the mature rosette stage were used in herbivore experiments.

Insect Colony. Beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), originally obtained from Bio-Serv, were reared for multiple generations on an artificial wheat germ-based diet (Bio-Serv) under the following conditions: 16 h/8 h light/dark; 28.5 °C. Adult moths were allowed to mate and the eggs were collected to maintain the colony. To prepare for the experiment, a subset of early fourth instar caterpillars were selected for cauterization to impair salivary secretions as outlined below.

Spinneret Cauterization. Cauterization of caterpillar spinneret prevents labial salivary secretion but does not affect insect feeding.[8,10] Early fourth instar caterpillars were cooled on ice and their spinneret cauterized using a hot probe. After a recovery period of 2−4 h, all caterpillars, both cauterized and mock-cauterized, to be used in the experiment were tested for labial salivary secretions by the glucose oxidase assay.[44] Briefly, individual caterpillars were restricted in a medicine cup containing a glass microfiber disk (Whatmann) saturated with a glucose/sucrose solution (50 mg/mL of each solution, Sigma); sucrose stimulates feeding and glucose is a substrate for the GOX reaction. After evidence of feeding, the disk was incubated

with 150 μg of 3,3′-diaminobenzidine tetradehydrochloride (1 mg/mL, pH 5.8, Sigma) and 2.5 U horseradish peroxidase (Sigma) in 50 mM potassium phosphate (Sigma), pH 7.0. The presence of a dark brown precipitates indicates that the caterpillar was able to secrete salivary GOX. Evidence of feeding but the absence of the dark brown precipitate indicates that the cauterization was successful. After confirmation of enzyme activity, caterpillars were placed on *Arabidopsis* "feeder" plants for approximately 24 h to allow them to acclimate to a plant diet before the experiment.

Experimental Design. Three days before the experiment, plants were enclosed in plastic containers with netting secured to the top to prevent caterpillar escape. Twenty-four plants were randomly assigned into four groups: control, mechanically wounded, herbivory by caterpillars with intact or with impaired salivary secretions. To prevent volatile signaling, plants were physically separated by plastic bins (dimensions: 55 cm × 39 cm × 28 cm ($l \times w \times h$)). At 4 pm, plants were treated: one subset of plants (wounded) had approximately 25% of leaf material removed by circular cuts with care taken not to cut the leaf midvein to mimic caterpillar herbivory. The second and third subset of plants had three-fourth instar caterpillars with either intact or impaired salivary secretions, respectively, added to each plant.. After 18 h, rosette leaves that showed signs of damage (wound, caterpillar ± salivary secretions) and all rosette leaves from control plants were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Plant Protein Extraction and SDS−PAGE. Leaf tissue (1 g) was ground in liquid nitrogen and thawed in 4 mL of helium-purged homogenization buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton, 5% glycerol, protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl floride, bestatin, pepstatin A, E-64, leupeptin and 1,10-phenanthroline (1:30, v/v) (Sigma)). Cell wall, nuclei, chloroplasts, and debris were removed by two centrifugations at 4000*g* at 4 °C for 10 min. The supernatant was then centrifuged at 30000*g* at 4 °C for 30 min yielding the soluble protein fraction. Protein concentration was measured using a Bradford assay (Bio-Rad) using bovine serum albumin as standard.[45] Soluble protein (120 μg) was diluted 1:5 in protein dissociation buffer (0.5 M Tris-HCl, pH 6.8, 25% glycerol, 10% w/v sodium dodecyl sulfate, 1% w/v bromophenol blue, 5 mM tris(2-carboxyethyl)phosphine and separated on a 11% SDS−polyacrylamide gel.[46] Coomassie-stained bands from 1D acrylamide gel were excised at 40−60, 60−80, and 80−100 kDa.

Protein Digestion and Nanoliquid Chromatraphy Electrospray Mass Spectroscopy/Mass Spectroscopy (LC/ESI/MS/MS). Following destaining in 50% methanol, gel slices were exposed to 10 mM dithiothreitol for 1 h at 56 °C; this treatment will reduce cysteine-associated sulfenic modifications.[47] Samples were then alkylated in 55 mM chloroacetamide for 1 h at room temperature. After being washed in 50 mM ammonium bicarbonate, the gel pieces were shrunk in 100% acetonitrile (ACN). Protein digestion was performed using 1700 U trypsin in 50 mM ammonium bicarbonate for 8 h at 37 °C under orbital rotation (600 rpm). After peptide extraction in 90% ACN in 0.5 M urea, samples were dried in a speed vac and resolubilized in 5% ACN in 0.2% formic acid (FA). Peptides were separated on a C_{18} column (150 μm × 10 cm) using an Eksigent nano liquid chromatography-2-dimensional system. Peptides were eluted using a linear gradient from 10 to 60% ACN containing 0.2% FA over 56 min at a flow rate of 600 nL/min.

The column was connected to a nanoprobe interfaced to a LTQ-Orbitrap mass spectrometer (ThermoScientific). Each full mass spectrum (MS) acquired at a resolution of 60 000 in the orbitrap was followed by three MS/MS spectra (four scan events) where the three most abundantly multiple charged ions were selected for MS/MS sequencing. Tandem MS experiments were acquired using collision-induced dissociation in the linear ion trap.

Peptide Analysis. Peak identification was conducted using Mascot version 2.2 (Matrix Science) against the *Arabidopsis* sequence database. The data were processed with tolerance parameters set to 0.03 and 0.5 Da for the precursor and the fragment ions, respectively. For protein sequence identification, the following variable modifications were set: oxidation (M), deamidated (NQ), phospho (STY), nitrosyl (C), sulfide (C), carbamidomethyl (C), 4-ONE (C), and sulfo (C). More than 400 proteins were analyzed for post-translational modifications (Supplementary Table S1, Supporting Information).

Results and Discussion

Salivary-Specific Modifications. Proteins in this category exhibit different modifications depending on if the plants were damaged by caterpillars with intact or impaired salivary secretions. If the expected modification induced in response to mechanical damage or herbivory by caterpillars with impaired labial salivary secretions is not observed in plants damaged by caterpillars with normal saliva, this suggests that a salivary-specific effector is preventing plant-induced responses. Alternatively, there may be an effector-specific alteration of plant responses which is only seen in the caterpillar-damaged plants.

Lipoxygenase 2. *Arabidopsis AtLOX2* (At3g45140; EC 1.13.11.12) encodes a 13S-lipoxygenase, a constitutive leaf protein that is strongly induced by increases in endogenous JA that occur in response to tissue damage.[48] This enzyme processes chloroplast-derived α-linolenic acid to the 13(*S*)-hydroperoxyoctadecatrienoic acid, which represents the first committed step of octadecanoid biosynthesis.[49]

In this study, two AtLOX2 proteins were identified; one constitutively expressed (MW 98 975.46) and the other only in wounded tissues (MW 101 898.94), both mechanically damaged and insect-damaged. The observed size difference represents the amino-terminal 26 amino acid transit peptide on prechloroplast proteins.[50] This supports our understanding of LOX2 regulation; in response to stress, constitutive levels lead to an increase in JA production which is an upstream signal for *AtLOX2* gene expression, leading to a rapid feedforward activation of plant defense responses; therefore, in the stressed plants, the induced, preprocessed form of LOX2 is present.

In response to wounding, *AtLOX2* is rapidly induced;[42,51] however, even though a salivary-specific difference in *LOX2* expression was not observed in plants fed upon by caterpillars with intact or with impaired salivary secretions, JA levels were higher in plants damaged by caterpillars with impaired salivary secretions.[9] This suggests there is post-translational regulation of the JA-biosynthetic pathway.[3] In this study, the constitutively phosphorylated AtLOX2 Ser_{600} is dephosphorylated in response to damage by insects with normal salivary secretion (Figure 1). Studies are presently ongoing to determine if this covalent modification negatively affects AtLOX2 activity which may explain the lower JA levels in response to herbivory by caterpillars with normal salivary secretions.

Chaperonin. The constitutive ATP-dependent chaperonin (At3g18190) is phosphorylated on Thr_{59} and nitrosylated on

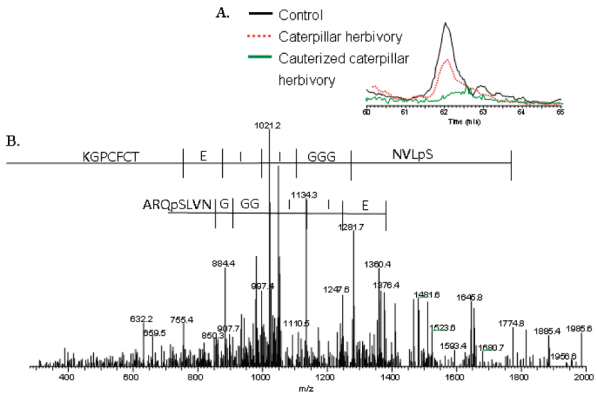

Figure 1. MS/MS spectrum of lipoxygenase2 phosphopeptide ARQSLVNGGGIIETCFCPGK (*m/z* 1065.99, 2^+). (A) Extracted ion chromatograph for *m/z* 1065.99 with (B) its corresponding MS/MS spectrum indicating phosphorylation of Ser_{600}. This protein is dephosphorylated in response to herbivory by fourth instar *S. exigua* caterpillars indicating a caterpillar-salivary specific modification.

Cys_{407} (Figure 2). In response to herbivory by caterpillars with intact salivary secretions, a 3-fold decrease in phosphorylation on Thr_{59} is observed compared to control plants and other treatments. In addition, this protein is nitrosylated on Cys_{407} in control plants and caterpillar-treated plants but denitrosylated in wounded plants or those fed upon by caterpillars with impaired salivary secretions. This suggests that salivary effectors may be responsible for activating a phosphatase and preventing wound-associated denitrosylation from occurring.

Caterpillar-Specific Modifications. Despite similarities in plant responses to mechanical damage and caterpillar herbivory, there are also striking differences which reflect the plants' ability to target their defense responses against the attacker.[1,3] The type and timing of wounding caused by caterpillar herbivores compared to mechanical damage and the presence of elicitors in the caterpillar regurgitant which may be transferred to the plant tissue during feeding are, in part, responsible for these distinctions.[5] The following section highlights caterpillar-dependent protein modifications observed in this study.

Transcriptional Repressor. The plastid transcriptional repressor (At4g18810) contains a predicted NmrA-like domain which allows it to behave as a redox sensor monitoring the changing NADPH/$NADP^+$ status and has been compared to the UV-B and ozone similarly regulatory protein (UOS1) or to a component of the photosynthetic NADH:ubiquinone oxidoreductase complex.[52,53] *PsUOS1* transcriptional levels decrease when *Pisum sativum* plants were exposed to low UV-B levels or ozone exposure.[54] Similar transcriptional and metabolic responses have been noted between plant response to caterpillar herbivory and to UV-B stress,[55,56] lending credibility to the involvement of this transcriptional regulator in plant responses to stresses. In this study, phosphorylation of Ser_{539} on this putative transcriptional repressor decreased in response to herbivory, suggesting that in response to biotic stress regulation of this protein may occur both transcriptionally and post-transcriptionally (Figure 3).

DNA-Repair ATPase-Related Protein. The constitutive DNA-repair, ATPase-related protein (At2g24420) is modified in a caterpillar-specific manner. Expression of this protein has been observed to respond to abiotic stresses, such as drought through a DREB-1A-independent pathway.[57] Since there are

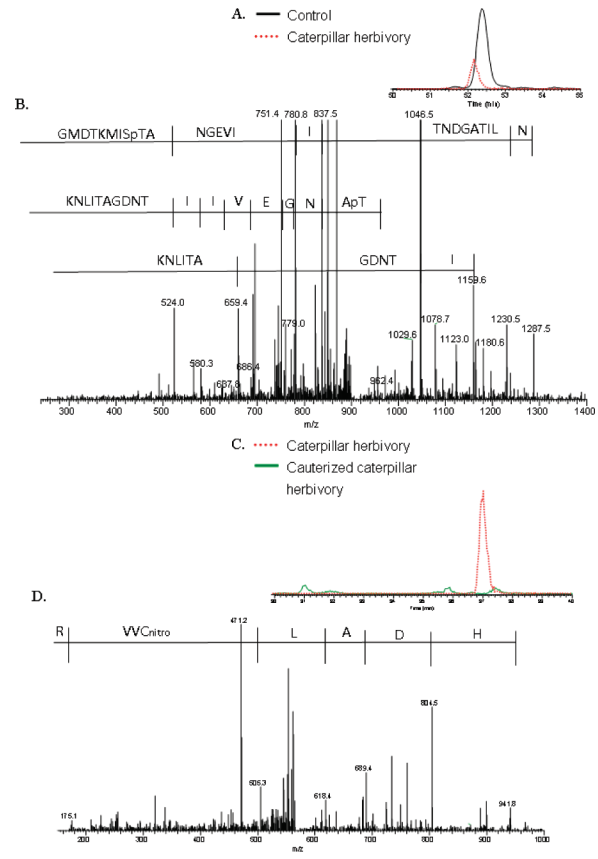

Figure 2. MS/MS spectrum of chaperonin (At3g18190) phosphoprotein GMDKMISTANGEVIITNDGATILNK (*m/z* 907.19, 3^+) and tryptic peptide SLHDALCVVR (*m/z* 381.23, 3^+). (A) Extracted ion chromatograph for *m/z* 907.19 with (B) its corresponding MS/MS spectrum indicating phosphorylation of Thr_{59}. In response to herbivory by caterpillars with normal salivary secretions, dephosphorylation is observed, suggesting a salivary-specific protein modification. (C) Extracted ion chromatogram for *m/z* 381.23 with (D) its corresponding MS/MS spectrum indicating nitrosylation of Cys_{407}. Wound-specific denitrosylation (inset) is not observed in caterpillar-damaged plants.

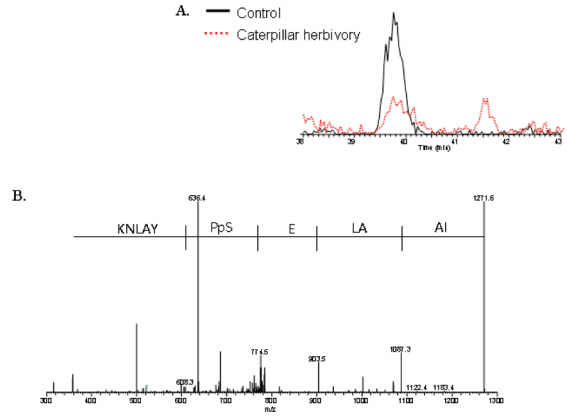

Figure 3. MS/MS spectrum of transcriptional repressor (At4g18810) phosphopeptide ICIAALESPYALNK (*m/z* 793.40, 2^+). (A) Extracted ion chromatograph for *m/z* 793.40 with (B) its corresponding MS/MS spectrum indicating phosphorylation of Ser_{539}. In response to herbivory, caterpillar-specific dephosphorylation is observed.

noted similarities in plant responses to herbivore feeding and water stress, this protein may be involved in plant responses to these stresses.[51,58]

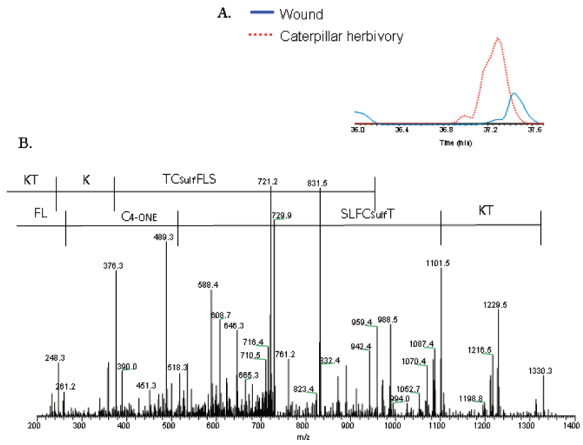

Figure 4. MS/MS spectrum of a DNA-repair, ATPase-related protein (At2g24420) FLCSLFCTKTK (*m/z* 738.88, 2^+). (A) Extracted ion chromatograph for *m/z* 738.88 with (B) its corresponding MS/MS spectrum indicating 4-oxo-2-nonenal peroxidation of Cys_{411} and oxidation of Cys_{415}. In wounded plants, the protein is not modified. In response to caterpillar herbivory, Cys_{411} undergoes 4-oxo-2-nonenal peroxidation and Cys_{415} is oxidized to the sulfinic acid state.

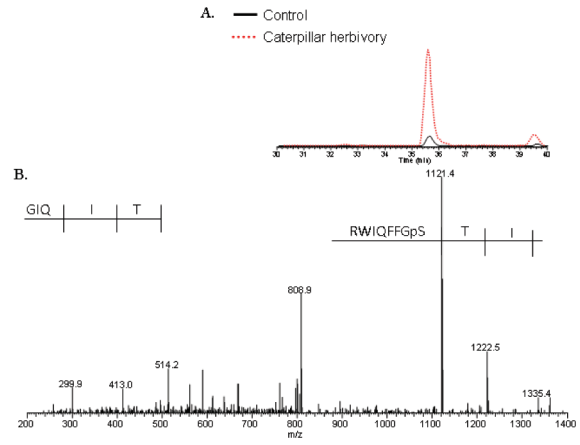

Figure 5. MS/MS spectrum of photosystem I P700 A1 apoprotein phosphopeptide GIQITSGFFQIWR (*m/z* = 817.90, 2^+). (A) Extracted ion chromatograph for *m/z* 817.90 with (B) its corresponding MS/MS spectrum indicating phosphorylation of Ser_{139}. The constitutive protein is not phosphorylated. In response to herbivory by fourth instar *S. exigua* caterpillars, Ser_{139} is phosphorylated.

In this study, two caterpillar-specific modifications to this constitutive protein are observed; the lipid peroxidation of Cys_{411} and the oxidation of Cys_{415} (Figure 4). Lipid hydroperoxides may be formed through enzymatic or nonenzymatic pathways.[38,59−61] In response to leaf damage, the increased reactive oxygen species of the activation of chloroplast-associated lipoxygenases oxidize endogenous membrane lipids leading to peroxyl radicals. These highly reactive electrophiles then preferentially attack cysteine sulfhydryl groups dramatically affecting protein activity and, in severe cases, leading to protein aggregation.[61,62] In response to caterpillar herbivory, this DNA-repair, ATPase-related protein was modified by 4-oxo-2-nonenal (4-ONE) on Cys_{411}. In addition, Cys_{415} was oxidized to the sulfinic state. This state is more stable than sulfenic acid, which, in the presence of a cysteine thiol, is readily reduced forming a disulfide bridge.[21]

Caterpillar-Specific Modifications to Photosynthesis-Associated Enzymes. In response to caterpillar herbivory, plants reorganize their metabolism often by reducing primary metabolic processes, such as photosynthesis, to shunt resources into defensive pathways.[63] In particular, foliar herbivory negatively impacts photosynthesis[58,64] not only by removing photosynthetic biomass, but, in some cases, insect damage can affect photosynthetic processes in systemic, undamaged tissues.[65] In the following examples, caterpillar-specific changes to photosynthetic-associated enzymes are observed.

PS1 P700 A1 Apoprotein. In the chloroplast thylakoid membrane, photosystem I (PS I) P700-A and -B apoproteins and their associated chlorophyll molecules form the PS I reaction center that captures light energy to drive the second photosynthetic electron transport chain.[66,67] This constitutive protein exhibits caterpillar-associate increase in Ser_{139} phosphorylation (Figure 5). The effect of this modification on photosynthesis is unknown.

Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase. Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is a key plant enzyme as it catalyzes both photosynthetic carbon fixation and photorespiratory carbon oxidation.[68,69] This key regulatory enzyme is regulated at many levels, including transcriptionally, co- and post-transcriptionally, sugar-based inhibitors, allosteric effectors, and proteolytic degradation.[70−72] Disulfide linkages between Cys residues in the Rubisco large subunit is a highly conserved regulatory mechanism mediated through protein degradation and allows the protein to be in tune with changing chloroplastic redox conditions.[73,74] As light is detected by the photosynthetic machinery, the excitation and flow of electrons result in the production and distribution of reducing power and, subsequently, the activation of anabolic pathways. However, conditions of high oxidative stress results in the negative regulation of Rubisco. In a recent model proposed by Moreno et al,[75] as stromal oxidative potential increases, oxidation of Cys_{449} and Cys_{459} occurs, resulting in decreased activity. As oxidative stress continues, often to a detrimental point, oxidation of Cys_{172} leads to the formation of an intramolecular disulfide bridge with its thiol partner, Cys_{192}, which blocks the enzymatic catalytic site further inhibiting activity.[74,76] The change in Rubisco conformation also exposes a loop between Ser_{61} and Thr_{68} increasing the susceptibility of the protein to degradation.[77]

Other mechanisms that regulate Rubisco activity under increasing oxidative conditions include the attenuation of the dimerization of the large subunit which is needed for activity and translational attenuation.[78−80] In spinach and tobacco, Cys_{247} cross-links the large Rubisco subunits in the holoenzymes through disulfide bridges.[80,81] Oxidative stress can prevent the interactions between Rubisco large subunits or between the large subunit and chaperonins and cause conformational shifts that expose an N terminal-associated RNA recognition motif resulting in translational attenuation.[78,79]

In the leaves, Rubisco large subunit (gi:5881702) is constitutively expressed (Figure 6). In this study, Cys_{192} of control plants is oxidized to the sulfenic acid state. This form of cysteine is resilient to disulfide bridge formation and requires enzymes such as ATP-dependent sulphridoxins to be converted into the reduced form.[30,31] In comparison, a 5-fold reduction in Cys_{192} oxidation was seen in plants infested by *S. exigua* caterpillars. In plants treated with cauterized caterpillars, Cys_{192} was only present as the thiolate anion ($Cys\text{-}S^-$), which can react with Cys-SH to form a disulfide bridge, particularly in the presence

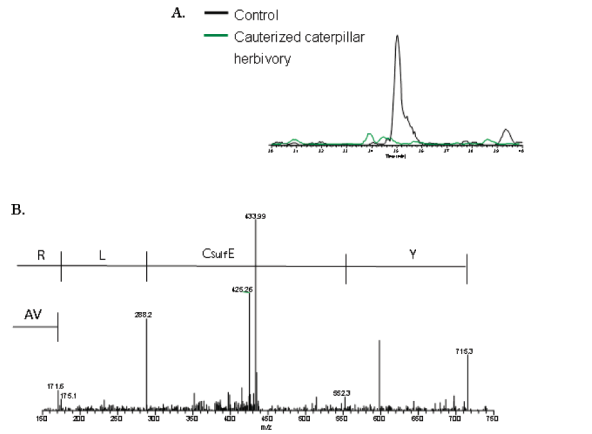

Figure 6. MS/MS spectra of Rubisco large subunit tryptic peptides AVYECLR (m/z = 443.21, 2^+). (A) Extracted ion chromatograph for m/z 443.21 with (B) its corresponding MS/MS spectrum indicating oxidation of Cys_{192}. In control plants, Cys_{192} is present as the oxidized sulfinic acid. In comparison, a lower level of Cys_{192} oxidation is observed in caterpillar-infested plants, and Cys_{192} is only present as the thiolate anion in plants eaten by cauterized caterpillars. Lower levels of oxidation lead to decreased enzyme activity and degradation.

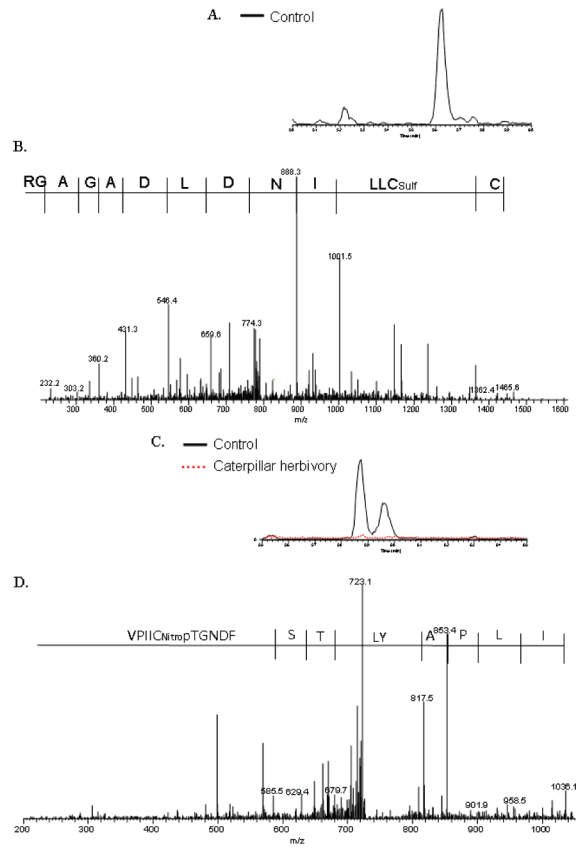

Figure 7. MS/MS spectra of Rubisco activase (At2g39730) tryptic peptides MCCLLINDLDAGAGR (m/z = 798.85, 2^+) and VPIICTGNDFSTLYAPLIR (m/z = 735.02, 3^+). (A) Extracted ion chromatograph for m/z 798.85 with (B) its corresponding MS/MS spectrum indicating oxidation of Cys 165 to the sulfinic acid. (C) Extracted ion chromatogram for m/z 735.02 with (D) its corresponding MS/MS spectrum indicating nitrosulation on Cys_{223} and phosphorylation on Thr_{224}. In response to caterpillar herbivory, dephosphorylation and denitrosylation of Rubisco activase are observed.

of plastid-associated thiol-disulfide oxidoreductases. Disulfide bond formation between Cys_{172} and Cys_{192} would result in Rubisco inhibition and lead to degradation. As predicted, in herbivore-infested plants, the functional (gi: 5881702) and N- and C-terminal processed form of Rubisco was detected (gi: 27752811).[82,83] This stress-related fragmentation may be enzymatic or nonenzymatically via ROS.[84−87]

Rubisco Activase. One level of Rubisco regulation is through the binding and inhibition by phosphorylated 5-C sugars, such as CA1P or ribulose 1,5-bisphosphate.[88] The ATP-dependent enzyme Rubisco activase (RCA) (EC 4.1.1.39; At2g39730) removes these inhibitors allowing photosynthesis to proceed. In *A. thaliana*, two RCA isozymes, 42 and 46 kDa, are generated through alternative splicing.[89] The larger RCA α-isozyme is sensitive to thioredoxin f-mediated redox regulation of Cys_{392} and Cys_{411}, key carboxy-terminus associated cysteine residues.[90−92] As light intensity increases, increased levels of reduced thioredoxin lead to the reduction of key cysteines on RCA. As a result, enzyme activity increases due to a decreased sensitivity to ADP inhibition. This close tie with redox regulation allows photosynthesis to be adjusted to ever changing light conditions. In comparison, the smaller isozyme exhibits a higher metabolic capacity (V_{max}).[93]

In this study, constitutive oxidation of Cys_{165} in one of the nucleotide binding domains, Walker B, was observed;[94] cysteine was observed to be in the sulfinic acid (Cys-SO_2H) state (Figure 7). In comparison, a caterpillar-specific decrease in nitrosylation of Cys_{223} and phosphorylation of Thr_{224} was observed; these amino acids are within the sensor I domain of RCA which are also involved in ATP binding.[95] To our knowledge, the physiological relevance of this Cys_{223} and Thr_{224} modification has not been investigated but may impair ATP-binding, thereby negatively influencing photosynthesis, in response to caterpillar herbivory.

Conclusion

In this study, *Arabidopsis* plants were exposed to herbivory by fourth instar *S. exigua* caterpillars that had intact or impaired salivary secretions, and the effects on protein covalent modifications were assessed. Both caterpillar- and salivary-specific post-translational modifications of plant proteins were observed; many of these changes were not previously identified. Caterpillar herbivory often results in a reallocation of plant metabolic resources and may be accompanied by a systemic stasis of photosynthesis.[58,63−65] In this study, three photosynthesis-associated proteins, PS I P700 apoprotein A1, Rubisco large subunit and Rubisco activase, exhibit caterpillar-specific modifications (Figures 5−7). Rubisco is constitutively expressed; in control plants, Cys_{192} is oxidized to the sulfinic acid state. In comparison, low levels of Cys_{192} oxidation is observed in herbivore-treated plants. Instead, Cys_{192} was present as the thiolate anion which may lead to decreased enzyme activity and, eventually, protein degradation.[75,76] For Rubisco activase, caterpillar-associated modifications were associated with protein domains involved in ATP binding, suggesting that nucleotide binding may be impaired. Therefore, caterpillar-associated post-transcriptional modifications of these two proteins may support observations at the whole plant level that chewing insect herbivory negatively impacts photosynthesis.[58,64,65] Other caterpillar-specific modifications include the dephosphorylation of the transcriptional regulator (At4g18810) and the lipid peroxidation and cysteine oxidation of the DNA-repair protein (At2g24420).

Two caterpillar labial salivary-specific modifications were observed: dephosphorylation of LOX2 and a chaperonin (At3g18190) (Figures 1 and 2). This suggests that an effector in the caterpillar labial saliva, such as the enzyme GOX, affects the cellular redox potential and activates a redox-dependent phosphatase, such as PP7.[40] This is the first report of the covalent modification of *Arabidopsis* LOX2; the effect of phosphorylation is unknown, but studies are ongoing to determine its role in plant-insect interactions.

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Supporting Information Available: Table of protein post-translational modifications. This material is available free of charge via the Internet at http://pubs.acs.org.

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